*Journal of Chromatography*, 512 (1990) 131–137
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1818

# Purification of a amylase–pullulanase bifunctional enzyme by high-performance size-exclusion and hydrophobic-interaction chromatography

CHEORL-HO KIM* and DONG-SOO KIM
*Laboratory of Biochemistry, Department of Agricultural Chemistry, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113 (Japan)*
HAJIME TANIGUCHI
*Laboratory of Bioconversion, National Food Research Institute, 2-1-2 Kannodai, Tsukuba 305 (Japan)*
and
YOSHIHARU MARUYAMA
*Laboratory of Biochemistry, Department of Agricultural Chemistry, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113 (Japan)*

## ABSTRACT

A novel bifunctional enzyme, amylase–pullulanase enzyme (APE), which is produced by *Bacillus circulans* F-2, was separated and purified in only two steps by high-performance size-exclusion (HPSEC) and high-performance hydrophobic-interaction chromatography (HPHIC) with 50 m*M* phosphate buffer (pH 7.3) containing 5 m*M* $Co^{2+}$. About a 90% recovery of the total enzyme activity was achieved, together with a 1821-fold increase in specific activity. APE activity recovered from the column decreased rapidly in the absence of $Co^{2+}$, which acts as an activator and stabilizer. However, most of the activity was restored on the addition of $Co^{2+}$. When a descending salt gradient (1 to 0 *M* ammonium sulphate in 60 min) and a mobile phase containing $Co^{2+}$ were used in HPHIC, the APE characteristics were altered, resulting in earlier elution of the enzyme. The results indicate that the hydrophobic properties of APE can be altered by the addition of $Co^{2+}$, and that the application of HPSEC and HPHIC with cations such as $Co^{2+}$ results in the effective purification of a high-molecular-weight enzyme.

## INTRODUCTION

There are two kinds of enzymes that can hydrolyse the α-1,6-glucano binding site on starch materials: an isoamylase (E.C. 3.2.1.68) and a pullulanase (E.C. 3.2.1.41). Whereas pullulanase hydrolyses only the α-1,6-glucano bonds of pullulan and amylopectin, isoamylse can hydrolyse the α-1,6-glucano bonds of amylopectin and glycogen, but not pullulan. These enzymes have been called debranching enzyme or

0021-9673/90/$03.50 

debranching amylase[1]. Pullulan, a very highly ordered and branched polysaccharide with a 2:1 ratio of α-1,4-glucano to α-1,6-glucano bonds, is usually produced by the bacterium *Pullularia pullulans*. There have been some reports of the enzymatic application and production of pullulanase[2–7], but the work was focused only on the enzymatic characteristics and the utilization of the pure pullulan molecules. The bifunctional enzyme which has an ability to digest starch (α-1,4-glucano linkage) and pullulan (α-1,6-glucano linkage) has become important in fermentation processes and academic fields.

Amylase–pullulanase enzyme (APE) (α-1,4-and α-1,6-glucanohydrolase) is a high-molecular-weight (*ca.* 220 kDa) extracellular protein of *Bacillus circulans* F-2[8]. Although APE is an abundant protein, it has proved difficult to isolate and purify because it is inactivated in the process of purification. Analysis of the purified APE showed that cobalt chloride activated and stabilized the APE activity.

In previous work[8], several extensive purification steps involving ammonium sulphate fractionation, starch adsorption, DEAE-Toyopearl chromatography and high-performance hydrophobic-interaction chromatography (HPHIC) were utilized to purify APE. These methods are time consuming and produce low yields of purified APE, necessitating the use of large amounts of starting material.

The purification scheme illustrated in this study provides a simple rapid and quantitative method of isolating APE from bacterial culture supernatants. Further, it is shown that application of HPHIC with reagents such as cobalt chloride permits separation.

## EXPERIMENTAL

### *Isolation of amylase–pullulanase enzyme*

The bacterial strain used was described previously[9]. The strain was cultivated in a 5-l jar fermenter (Marubishi, Tokyo, Japan) containing 1 l of the medium with the composition described previously[8]. Cultivation was carried out for 4 days at 37°C while rotating the vessel at 150 rpm. The bacterial culture supernatant was concentrated with ammonium sulphate (0–80%) and the resulting enzyme was injected onto high-performance liquid chromatographic (HPLC) columns.

### *Chemicals*

Soluble starch purchased from E. Merck (Darmstadt, F.R.G.) was reduced with sodium borohydride and used in amylase assays as the substrate. Pullulan (MW 50 000) was purhased from Hayashibara Biochemical Labs. (Tokyo, Japan), cobalt chloride and sodium borohydride from Fisher Scientific (Louisville, KY, U.S.A.), TSK gel G3000 SW-XL and TSK gel phenyl 5PW from Tosoh (Tokyo, Japan) and HPLC-grade ammonium sulphate and protein standards for molecular weight measurement from Bio-Rad Labs. (Richmond, CA, U.S.A.).

### *Protein assay*

The protein concentration of the column eluates was monitored by measuring the absorbance at 280 nm, and those of the enzyme solution and other proteins were measured by the method of Lowry *et al.*[10] using bovine serum albumin as the standard.

*Assay of amylase and pullulanase activities*

Amylase and pullulanase activities were measured by the method used previously[8] and that of Somogyi[11]. One unit of the enzyme was defined as the amount of enzyme that produced reducing sugars corresponding to 1 mmol of glucose from soluble starch or pullulan in 1 min under the assay conditions. Specific activity was expressed as the units per milligram of protein.

*Gel electrophoresis*

Zymograms of amylase activity staining were obtained with a previously reported method[1]. Native slab polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Davis and Ornstein[12] with 7.5% polyacrylamide gel at pH 8.3. Sodium dodecyl sulphate (SDS) PAGE was done by the procedure of Weber and Osborn[13] with 12.5% polyacrylamide gel. For relative molecular mass measurement, myosin ($M_r$ 205 000), $\beta$-galactosidase ($M_r$ 116 000), phosphorylase B ($M_r$ 97 400), bovine serum albumin ($M_r$ 66 000) and egg albumin ($M_r$ 45 000) were used as standards.

*High-performance size-exclusion chromatography (HPSEC)*

An analytical size-exclusion column (TSK gel G3000 SW-XL), particle size 10 $\mu$m (300 × 7.5 mm) (Tosoh), was used for amylase–pullulanase separation, as described previously[8]. HPLC was performed at ambient temperature with a Shimadzu (Tokyo, Japan) solvent-delivery module, including a Model LC-4A system controller and injector block. Concentrated culture broth was applied in 250–500-$\mu$l volumes with a Hamilton syringe. The elution buffer (pH 6.5) was 50 m*M* phosphate buffer–0.3 *M* sodium chloride. All buffers were filtered through a 0.45-$\mu$m filter (Millipore). Elution was carried out at a flow-rate of 0.5 ml/min. Fractions were collected at 0.5-min intervals in 75 × 12 mm tubes. Recoveries were in the range 95–100%.

*HPHIC*

Chromatography was performed at ambient temperature. All buffers were vacuum filtered through Millipore (Bedford, MA, U.S.A.) 0.45-$\mu$m HAWP filters before use. A volume of 300 $\mu$l of the APE activity eluted from the HPSEC, to which 1.0 *M* ammonium sulphate had been added, was pumped at 1 ml/min into a hydrophobic-interaction HPLC column (21.5 × 150 mm) of TSK gel phenyl 5PW (Tosoh) previously equilibrated with 1.0 *M* ammonium sulphate–50 m*M* sodium phosphate (pH 7.3) (buffer A). Elution was carried out with a Shimadzu solvent-delivery module including a Model LC-4A system controller and injector block. All samples were adjusted to 1.0 *M* ammonium sulphate prior to injection.

Unless stated otherwise, the gradient programme consisted of a preliminary wash with buffer A at a flow-rate of 1 ml/min, then, following sample injection, a descending salt gradient was developed with 50 m*M* sodium phosphate (pH 7.3) (buffer B) in 60 min. Buffer B was then maintained at a flow-rate of 1 ml/min for the next 20 min before re-equilibration with buffer A. In experiments that required cobalt chloride in the mobile phase, both buffers A and B contained 5 m*M* cobalt chloride.

Fractions of 0.4 ml were collected and the amylase and pullulanase activities were determined. The recoveries of total enzyme activity and injected protein were almost always 90–100%.

## RESULTS AND DISCUSSION

APE, a bifunctional enzyme produced by *Bacillus circulans* F-2, possesses two active sites which hydrolyse α-1,4-and α-1,6-glucosdic linkages at the same rate[8]. APE is a very minor component of the *B. circulans* F-2 amylase system and its physiological function is not known[1,8]. Thus, a sensitive method is required for its purification in studies of its structure–function relationship.

A previous study of the purification of APE from culture broth included fractonation with ammonium sulphate, DEAE ion-exchange chromatography and chromatofocusing[8]. It was shown that the purified APE activity was high and stabilized in the presence of cations such as $Co^{2+}$. In this study, HPSEC and HPHIC methods were selected to optimize the recovery of purified APE, as the ammonium sulphate fractionation greatly reduced the activity of the crude enzyme and APE is a high-molecular-weight (220 kDa) protein. In this paper data are presented to show the influence of an enzyme-modifying and -stabilizing reagent on APE.

### *HPSEC*

The first step in the purification of APE from culture broth was HPSEC. The procedure not only gave a 66-fold (amylase) and 844-fold (pullulanase) purification of the specific activity, but also yielded 7.4% (amylase) and 94.1% (pullulanase) recoveries of total activity.

Fig. 1 is a representative HPLC elution pattern of the concentrated crude enzyme obtained under the conditions described above and the zymogram of APE on the native PAGE after successful HPSEC, demonstrating the purification achieved. As shown in Fig. 1A, the resolution obtained by HPLC was excellent, indicating satisfactory separation of APE from other proteins.

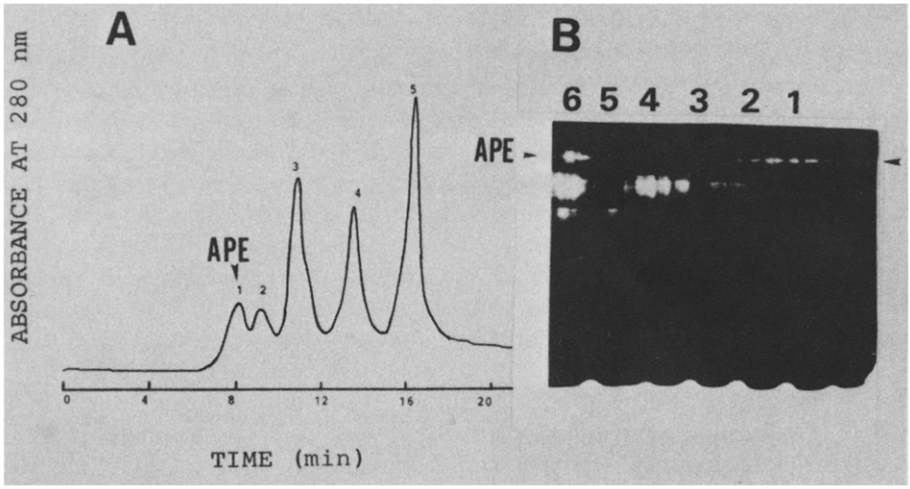


Fig. 1. Separation of APE (peak 1) from culture supernatant by HPSEC. (A) Column, TSK gel G3000 SW-XL; eluent, 50 m*M* sodium phosphate–0.3 *M* sodium chloride (pH 6.5); flow-rate, 0.5 ml/min; temperature, 25°C. (B) Native PAGE zymogram of samples (A) from HPSEC of bacterial culture supernatant; lane 1 = peak 1 (arrow denotes position of APE); lane 2 = peak 2; lane 3 = peak 3; lane 4 = peak 4; lane 5 = peak 5; lane 6 = starting material.

After collection of the peaks the APE activity was determined. Peak 1 was found to contain catalytically active APE. For comparion, lane 6 in Fig. 1B contains the crude enzyme solution before chromatography. Activity-stained PAGE showed that peak I has a single activity band. Peaks 3, 4 and 5 show no APE band, and peak 2 shows a small APE band. The APE-containing peak shows a retention time of 8.10 min, corresponding to a molecular weight of 200–220 kDa, consistent with that found for ADE isolated using DEAE ion-exchange chromatography in our laboratory and with the value reported previously[8]. This result indicates that the successful separation of APE from other carbohydrases can be achieved by HPSEC even though some impurities were still present.

*HPHIC*

The active APE (300 $\mu$l) from the above step was applied to a hydrophobic interaction column of TSK gel phenyl 5PW previously equilibrated with 1 *M* ammonium sulphate and 50 m*M* phosphate buffer (pH 7.5). APE was eluted from the HPHIC column at 22.32 min (corresponding to fraction No. 45), and was a single peak based on activity and protein measurements (Fig. 2A).

In previous work[8] we observed that cobalt chloride enhanced the APE activity.

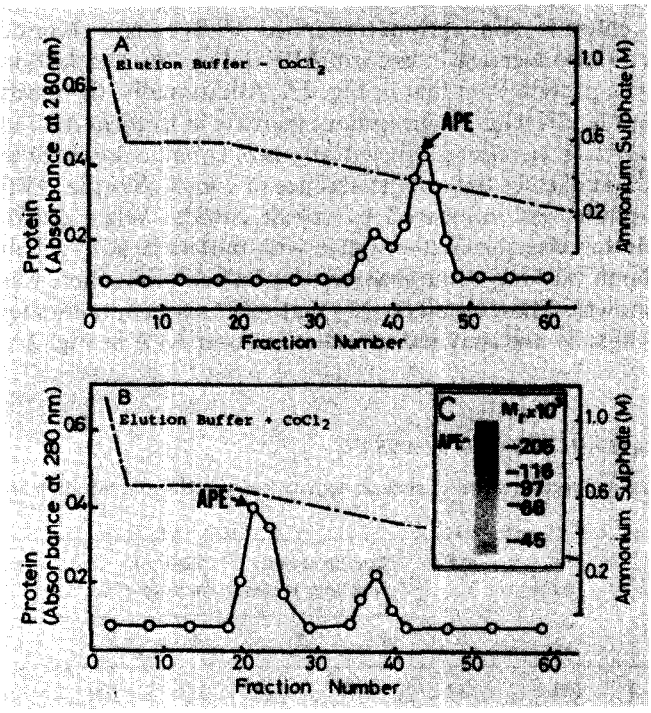


Fig. 2. Isolation of APE by HPHIC and influence of cobalt chloride on the hydrophobic characteristics of APE. Peak 1 of HPSEC was prepared in elution buffer A, as described in under Experimental. One set of aliquots was then made 5 m*M* with respect to cobalt chloride. The elution buffers in each instance are indicated. Purified APE of B was subjected to SDS-PAGE (12.05% gel) with molecular-weight markers. A, Without cobalt chloride; B, with cobalt chloride; C, SDS-PAGE of APE from B. $M_r$ = Molecular weight.

TABLE I

EFFECT OF COBALT CHLORIDE ON APE ACTIVITY

APE samples were stored at 4°C for up to 14 days after HPHIC in the appropriate buffer with or without cobalt chloride as described under Experimental.

| *Days after HPHIC* | *APE activity (%)*[a] | |
|---|---|---|
| | *Without $CoCl_2$* | *With $CoCl_2$ (5-mM)* |
| 0 | 100 | 100 |
| 1 | 80 | 100 |
| 2 | 50 | 98 |
| 3 | 40 | 95 |
| 7 | 20 | 90 |
| 14 | 10 | 80 |

[a] The APE activities given are the means of the amylase + pullulanase activities.

In contrast to the chromatogram shown in Fig. 2A (peak of APE, in the presence of cobalt chloride we observed a new peak (peak APE in Fig. 2B), which is eluted at 10.08 min (corresponding to fraction No. 21). When the APE-containing solution was adjusted to 5 m*M* cobalt chloride and chromatographed with cobalt chloride containing buffer, there was a sharp increase in isoform APE in Fig. 2B. The APE in Fig. 2B was a less hydrophobic protein than that in Fig. 2A. Additionally, there was a decrease in isoform APE in Fig. 2A with a concomitant increase in isoform APE in Fig. 2B when chromatograhed with increasing concentrations of cobalt chloride (0 to 10 m*M*) (data not shown). This indicates that, in the absence of cobalt chloride, APE attached to the stationary phase was influenced by cobalt cloride. When cobalt chloride-containing APE solution was chromatographed with buffers lacking cobalt chloride, using extensive column washing in the absence of cobalt chloride, there was little conversion (data not shown). These results imply that APE may contains sites that are sensitive to cobalt chloride and may indicate that isoform APE in Fig. 2A,

TABLE II

PURIFICATION OF APE FROM *BACILLUS CIRCULANS* F-2

Details of the purification procedure and measurement of enzyme activity and protein concentration are given under Experimental.

| *Purification step* | *Total protein (mg)* | *A*[a] *(units)* | *P*[a] *(units)* | *A/P* | *Specific activity (units/mg protein)* | | *Yield of activity (%)* | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *A* | *P* | *A* | *P* |
| Culture broth | 2250 | 2025.4 | 168.2 | 12.1 | 0.90 | 0.075 | 100 | 100 |
| TSK gel G3000 SW-XL | 2.5 | 149.1 | 158.2 | 0.94 | 59.6 | 63.3 | 7.4 | 94.1 |
| TSK gel phenyl-5 PW | 0.87 | 143.8 | 153.6 | 0.93 | 125.1 | 133.6 | 7.1 | 91.3 |

[a] *A* = Amylase activity; *P* = pullulanase activity.

which is modified by cobalt chloride, may be changed to a new conformation (isoform APE in Fig. 2B) resulting in different hydrophobicity.

Fig. 2C is an SDS-PAGE pattern demonstrating the purification achieved. The molecular weight of the APE band is about 220 kDA, which is the same as reported previously[8]. The specific activities (125.1 units/mg for amylase and 133.6 units/mg for pullulanase) of the HPSEC- and HPHIC-purified APE compare favourably with previously reported values (81.7 units/mg for amylase and 84.2 units/mg for pullulanase)[8]. Within 2 days after elution from the HPHIC column, the total enzyme activity recovered from the column, which eluted in the absence of cobalt chloride, decreased from 98% to 50% of the applied sample. However, the enzyme activity was maintained without any decrease in the total activity in the presence of 5m*M* $CoCl_2$, an activator and stabilizer of APE (Table I). Therefore, the loss of activity may be due to the absence of cobalt chloride. The results of these simpler and improved purification procedures are summarized in Table II.

## CONCLUSIONS

Purification of APE by HPSEC and HPHIC avoids the use of ammonium sulphate fractionation, which greatly reduces the activity of the crude enzyme. The method is rapid and the enzyme can be recovered in good yield. It is remarkable that the enzyme activity recovered (93%) in the final purification stage is about three times higher than the previous level (31%)[8]and is 1780-fold purified (as pullulanase) from the crude culture supernatants of *B. circulans* F-2. The present study also suggests that two isoforms are separated on the basis of different hydrophobic properties.

## ACKNOWLEDGEMENTS

This research was supported in part by Toyobo Biotechnology Research Foundation (Tokyo, Japan).

## REFERENCES

1 H. Taniguchi, in H. Taniguchi, S. Ohnish and Y. Sakano (Editors), *Amylase, Its Biotechnological Approach*, Japanese Academic Press Centre, Tokyo, 1986, p. 303.
2 H. Bender and K. Wallenfels, *Biochem. J.*, 79 (1961) 334–340.
3 M. Abdullah and D. French, *Arch. Biochem. Biophys.*, 137 (1970) 483–493.
4 S. Ueda and R. Ohba, *Agric. Biol. Chem.*, 36 (1972) 2381–2391.
5 G. J. Waker, *Biochem. J.*, 108 (1968) 33–40.
6 N. Nakamura, K. Watanabe and K. Horikoshi, *Biochim. Biophys. Acta*, 397 (1975) 188–193.
7 Y. Sakano, M. Higuchi and T. Kobayashi, *Arch. Biochem. Biophys*, 153 (1972) 180–185.
8 H. Sata, M. Umeda, C.-H. Kim, H. Taniguchi and Y. Maruyama, *Biochim. Biophys. Acta*, 991 (1989) 3388–394.
9 H. Taniguchi, C. H. Jae, N. Yoshigi and Y. Maruyama, *Agric. Biol. Chem.*, 47 (1983) 511–519.
10 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
11 M. J. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
12 B. J. Davis and L. Ornstein, *Ann. N.Y. Acad. Sci.*, 121(1964) 404–427.
13 J. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.